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A viral transcriptional activator of Kaposi's sarcoma-associated herpesvirus (KSHV) induces apoptosis, which is blocked in KSHV-infected cells

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Abstract

Replication and transcription activator (RTA), mostly encoded by Kaposi's sarcoma-associated herpesvirus (KSHV) open reading frame 50, is expressed in the immediate-early phase of reactivation and plays a critical role in inducing the viral lytic cycle in KSHV-infected cells. We established cell clones from BJAB cells and replication-deficient BCBL-1 cells in which KSHV RTA expression was controlled by an inducible promoter of the tetracycline-based Tet-Off expression system. In RTA-inducible BJAB cells, tetracycline removal induced the synthesis of RTA, resulting in cell death. DNA fragmentation, structural changes in the cell membrane, and poly(ADP-ribose) polymerase (PARP) cleavage were observed in the RTA-induced BJAB cells, indicating that RTA expression induced caspase activation and cell death by apoptosis. However, expression of RTA in RTA-inducible BCBL-1 cells did not undergo apoptosis and cell death. These results suggested that KSHV RTA is an apoptosis inducer that is opposed by an antiapoptotic pathway in infected cells.

Keywords: KSHV/RTA; Apoptosis; Cell death; Tet-Off

Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV), also designated human herpesvirus 8 (HHV-8), was discovered in Kaposi's sarcoma (KS) (Chang et al., 1994), the most common AIDS-related malignancy. KSHV DNA is found in most cases in both AIDS-related and classical KS lesions (Schulz, 1998). Moreover, KSHV is also involved in the pathogenesis of multicentric Castleman's disease (MCD) (Soulier et al., 1995), and primary effusion lymphoma (PEL) (Cesarman et al., 1995a), suggesting that KSHV could be an oncogenic DNA virus. Based on sequence analyses (Russo et al., 1996), KSHV is classified as a new member of the gammaherpesvirus subfamily and has genetic similarity to herpesvirus saimiri (HVS), murine gammaherpesvirus 68 (MHV68), and Epstein–Barr virus (EBV), which are also thought to be oncogenic viruses (Efstathiou et al., 1990; Moore et al., 1996).

Several KSHV-infected cell lines have been established from PELs: the BC-1 cell line, which is coinfected with both EBV and KSHV (Cesarman et al., 1995b; Gaidano et al., 1996), and the BCBL-1 cell line, which is positive for KSHV but not EBV (Arvanitakis et al., 1996; Renne et al., 1996). Similar to the other gammaherpesviruses (zur Hausen et al., 1978), KSHV can be induced to undergo lytic replication by chemical agents such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and *n*-butyrate, in these cell lines (Miller et al., 1997; Yu et al., 1999).

Induction of the viral lytic cycle, which involves viral

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DNA replication and the release of virions, results in cell death in almost all cases, due to massive viral amplification followed by the destruction of cellular survival mechanisms. In the case of adenovirus, expression of an early gene, E1A, causes p53 induction, which leads to apoptosis (programmed cell death), and another early gene, E1B, antagonizes this process by inhibiting p53 activity and functioning as a Bcl-2-like molecule. Thus, the regulation of cell death is important for viral replication (Debbas and White, 1993). Consistent with this, herpes simplex virus type 1 (HSV-1) can induce or inhibit apoptosis in a cell type dependent manner (Galvan and Roizman, 1998), and KSHV has many genes that have pro- and antiapoptotic function (Cheng et al., 1997; Ojala et al., 1999; Belanger et al., 2001; Seo et al., 2001; Feng et al., 2002).

Immediate-early (IE) genes have a critical role in bringing about lytic replication. These genes, most of which encode regulatory proteins that alter the expression of viral and cellular genes, are the first class of viral genes expressed after primary infection or reactivation. Their expression leads to the upregulation of early and late genes and maturation of the virus particle. KSHV replication and transcription activator (RTA) is expressed very early (<4 h) in reactivation, prior to the other lytic genes (Sun et al., 1998; Lukac et al., 1999). Recent analyses have shown that the expression of the RTA gene, and not the K8 gene, can induce the viral lytic cycle in KSHV-infected cell lines (Lukac et al., 1999; Chen et al., 2000; Gradoville et al., 2000), and we reported that induction of some lytic gene expression and viral replication were markedly reduced in an RTA-depressed cell line that we had subcloned from BCBL-1 cells (Nishimura et al., 2001).

In this study, we established conditional RTA-expressing systems in both KSHV-infected and uninfected cells and used them to investigate the function of RTA. We found that RTA expression led to apoptosis induction in uninfected cells (BJAB) but not in infected cells (BCBL-1 and BC-3). These results suggest that RTA could be, not an apoptosis blocker, but an inducer like adenoviral E1A, and in infected cells, various gene products protect against this process to enable efficient viral amplification.

Results

Isolation of stable BJAB cell clones that inducibly produce KSHV RTA

To investigate the function of the viral transcriptional activator encoded by KSHV ORF50 (RTA), we sought to isolate cell lines that conditionally express RTA. First, we transfected cell lines with an RTA-expression vector many times (pcDNA3.1-ORF50-cDNA), followed by G418 selection to establish a stably RTA expressing cell line. However, no clones were obtained after the selection after all, perhaps because of the toxicity of the stable expression of RTA. Therefore, we used the artificial transcriptional regulation system developed by Gossen and Bujard. In this system, the presence of tetracycline (tet) in the culture medium prevents a chimeric transactivator (tetracycline-responsive transcriptional activator, tTA) from binding to a synthetic promoter, while the removal of tet induces the transcription of the gene placed under the control of this promoter within a few hours (Gossen and Bujard, 1992). Several KSHV-infected cell lines that have been established from PELs are genotypically B cells with their immunoglobulin gene rearrangement (Drexler et al., 1998); therefore, we chose to use BJAB cells, EBV-negative Burkitt's lymphoma cells, to establish cells expressing RTA under control of the Tet-Off system. In these cells, the other KSHV genes were ignored.

We first established cell lines that stably expressed tTA (BJAB/off) by the Rev/Tet system (Clontech) and then introduced the RTA gene under control of a promoter with a Tet-responsive element (TRE) (Fig. 1A), as described under Materials and methods. After selection and cell cloning, 28 clones that were resistant to both G418 and hygromycin were screened for the conditional expression of RTA by IFA, and 6-positive clones were isolated. Among these, the clone named BJAB/off + RTA-27 showed the highest induction of RTA, so we used this clone in subsequent experiments.

RTA expression induces cell death

A highly regulated expression of RTA was observed 12 h postinduction in BJAB/off + RTA-27 cells by Western blotting using a specific antibody against RTA, and there was no background RTA expression seen in uninduced cells subjected to the same analysis (Fig. 1B). RTA expression was observed in almost all the cells by immunofluorescence assay (IFA), although the level of expression varied (data not shown). After RTA induction by the removal of tet from the culture medium, no morphological change was apparent within a few days, but a decrease in the number of live cells was observed a week after induction (data not shown). To determine whether cell death was induced in the RTA-expressing cells, we examined the cells microscopically with trypan blue staining and calculated the cell viability. As shown in Fig. 2A, cell death started 3 days postinduction, and the viability decreased to less than 50% 1 week later, while the cells without RTA induction were almost all viable. Similar results were obtained for the other BJAB/off + RTA clones, but not BJAB/off + TRE cells, in which only TRE (Fig. 1A) was introduced instead of TRE-RTA (data not shown).

These findings indicated that cell death was induced by RTA expression in BJAB cells a few days after induction.

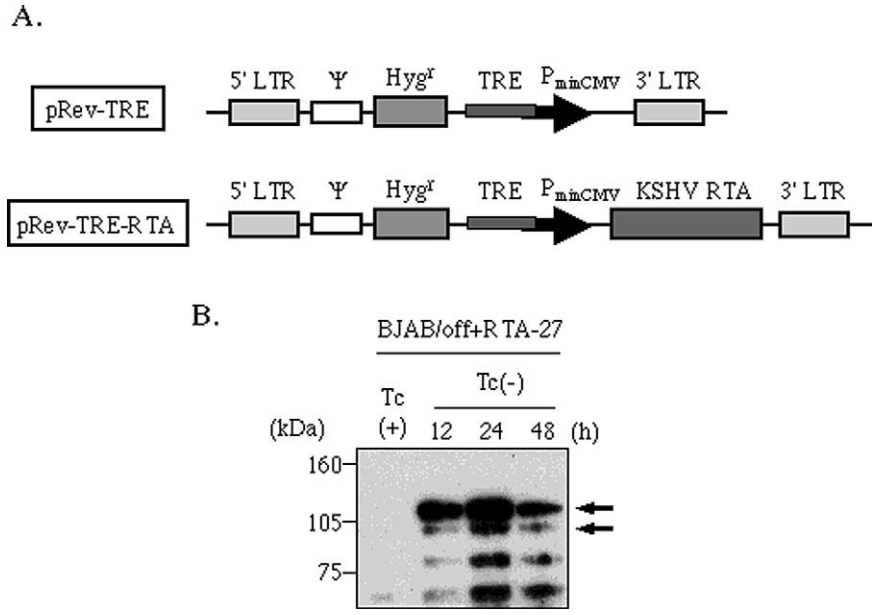


Fig. 1. Generation of stable BJAB clones that inducibly synthesize RTA. (A) Structure of retroviral vectors that contain the Tet-responsive element (TRE). TRE contains sevendirect repeats of the 42-bp tetO operator sequence, upstream of a minimal CMV promoter, which can be bound by the tTA transactivator: pRev-TRE is a control vector. pRev-TRE-RTA was constructed by inserting RTA cDNA into the *Hpa*I site of pRev-TRE. (B) RTA expression in BJAB/off + RTA-27 cells. Whole-cell extracts were obtained at different time points postinduction (Tc[−]) and analyzed by Western blotting using specific antibodies against RTA. The same amount of total protein (20 μg) was loaded in each lane. The positions of molecular mass markers are shown on the left. The arrow indicates RTA protein.

RTA expression induces cell death by apoptosis mechanism

To examine the cell death that was induced in RTA-expressing BJAB/off + RTA-27 cells, we tested for internucleosomal DNA fragmentation by the terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling (TUNEL) assay (Gavrieli et al., 1992). BJAB/off + RTA-27 cells with or without induction of RTA expression were harvested at different times and subjected to flow cytometric analysis as indicated under Materials and methods. The analysis clearly showed that DNA fragmentation occurred in the RTA-induced BJAB/off + RTA-27 cells 6 days postinduction (Fig. 3A), whereas no change was observed in uninduced BJAB/off + RTA-27 cells, even 8 days postinduction (data not shown). These results suggested that the RTA-induced death of BJAB/off + RTA-27 cells was caused by an apoptosis mechanism.

To further demonstrate that programmed cell death occurs in RTA-induced BJAB/off + RTA-27 cells, we analyzed the structural changes in the cell membrane. It is known that, soon after apoptosis, the membrane phosphatidyl-serine (PS) is translocated from the inner face of the plasma membrane to the outer surface. PS on the cell surface can be detected by Annexin V, which has a high affinity for it (Martin et al., 1995). Furthermore, propidium iodide (PI) does not penetrate cells in the early phase of apoptosis, but does in the late phase because of destruction of the cell membrane. As shown in Fig. 3C, the structural change had occurred in the membrane of RTA-induced BJAB/off + RTA-27 cells 4 days postinduction, and PI did not stain the cells at that time, but did 6 days postinduction (data not shown). These results suggest that 4 days after induction the cells were mostly in the early phase of apoptosis and by 6 days were mostly in the later phase, and that the RTA-induced cell death is caused by a canonical apoptosis mechanism.

In recent years, many of the molecules that participate in the highly ordered process of apoptotic cell suicide have been unveiled. The family of cysteine proteases known as caspases plays an essential role in apoptosis (Nicholson and Thornberry, 1997; Salvesen and Dixit, 1997). Caspases cleave several key structural cellular components, leading to the systematic and ordered disassembly of the dying cell. One of the best characterized caspase substrates is poly-(ADP-ribose) polymerase (PARP), a nuclear protein involved in DNA repair (Wang et al., 1997). We observed by Western blotting that PARP was cleaved in the RTA-induced BJAB/off + RTA-27 cells 4 days postinduction, but not in uninduced cells (Fig. 3E). This result suggested that caspases were activated in the RTA-induced BJAB/off + RTA-27 cells.

Taken together, the results from the TUNEL assay, Annexin V, PI staining, and Western blotting for PARP indicated that the expression of KSHV RTA induced the activation of a caspase signaling pathway, and as a result, the RTA-induced BJAB/off + RTA-27 cells died of apoptosis.

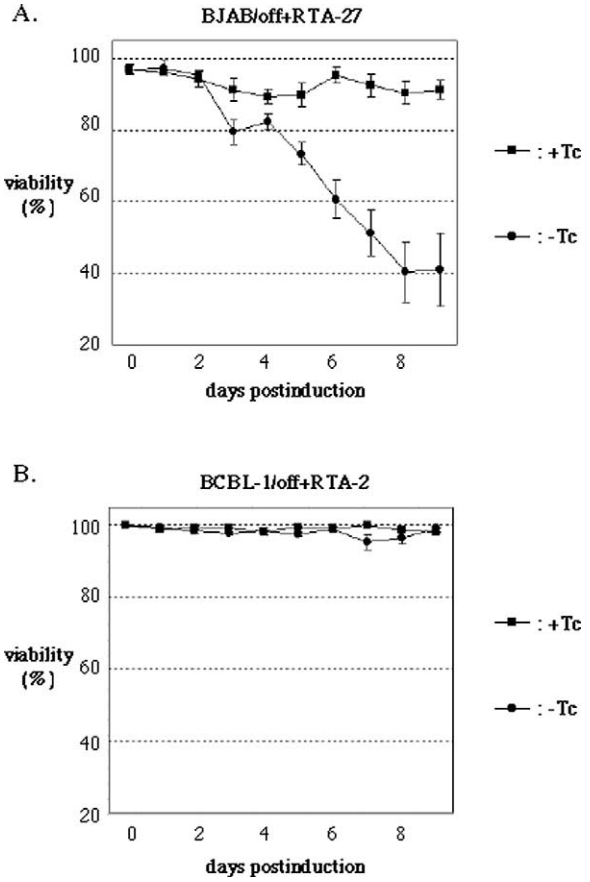


Fig. 2. Cell viability of RTA-induced cells. Induced (closed circles) or uninduced (closed squares) cells were collected at the indicated times and stained with trypan blue, followed by observation by microscopy. Cell viability was calculated by counting live and dead cells. A total of almost 300 cells was counted in each case. The points represent the average and the bars show the standard deviation (SD) of three independent transfections. (A) BJAB/off + RTA-27, (B) BCBL-1/off + RTA-2.

Isolation of RTA-inducible KSHV-infected cells

Since chemical agents, such as TPA and *n*-butyrate, that are used to induce lytic replication, can stimulate not only RTA expression but also many cellular and viral signaling pathways, we could not assess whether RTA expression induces the apoptosis in KSHV-infected cell lines, such as BCBL-1, BC-1, and BC-3. Therefore, we next tried to isolate cell clones from KSHV-infected cell lines, in which the RTA expression is regulated by the Tet-Off system, as in the BJAB/off + RTA-27 cells.

RTA protein is necessary and sufficient to drive the lytic cycle (Lukac et al., 1999; Nishimura et al., 2001), and RTA expression induces viral DNA replication and the release of mature viral particles that are toxic for host cells. We wondered if the apoptosis caused by RTA could not be distinguished from that due to viral proliferation. Recently, we reported that by subcloning BCBL-1 cells we isolated three RTA-depressed clones that did not respond to TPA induction and could not enter viral replication (Nishimura et al., 2001). From such subcloning, we also obtained replication-deficient clones. In one of these clones, the expression of immediate-early and early genes, for example the vIRF gene, were induced as in the parental BCBL-1 cells, but late genes, such as the K8.1 gene, were not induced (Fig. 4A) As a result, neither viral DNA replication (Fig. 4B) nor the release of viral particles (data not shown) took place in these replication-deficient cells. Thus, we used these replication-deficient KSHV-infected cells to create cells expressing RTA under the Tet-Off system and to exclude the effect of viral replication and particle production on the analysis of apoptosis induction by RTA.

We infected the replication-deficient BCBL-1 cells with a tTA-encoding retrovirus, followed by a TRE-RTA-encoding one, as the methods for creation of RTA-inducible BJAB clones, and isolated 24 clones that were resistant to both G418 and hygromycin. Among these, six positive clones were isolated by screening the induction of RTA with IFA, and of these, the one named BCBL-1/off + RTA-2 showed the highest induction of RTA, so we used this clone in the subsequent experiments.

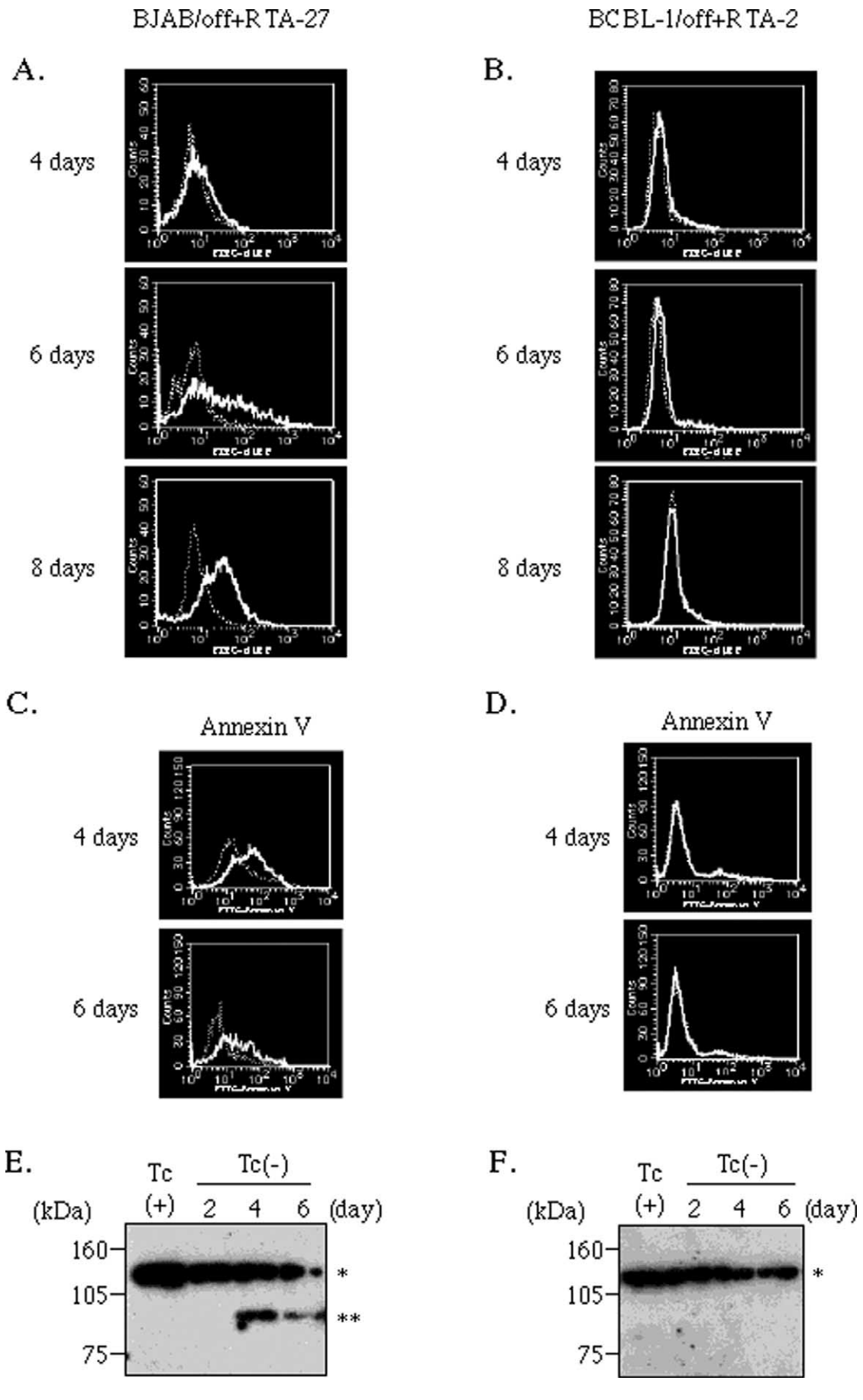
RTA expression induced other viral gene expression

Very low background and highly induced RTA expression was observed in the BCBL-1/off + RTA-2 cells by Western blotting (Fig. 4C). First, we assessed the induction of lytic gene expression followed by the RTA expression. As shown in Fig. 5, RTA induction by the removal of tet from the culture medium led to the expression of many lytic genes in BCBL-1/off + RTA-2 cells, similar to the effect of TPA. However, these did not include late genes because the parental BCBL-1 cells were replication-deficient cells in which no late gene expression was observed by TPA induction. This induction of early gene expression by RTA agreed with previous reports from other groups and ourselves (Sun et al., 1998; Lukac et al., 1999; Nishimura et al., 2001).

RTA expression did not result in apoptosis in KSHV-infected cells

In BJAB cells, RTA expression resulted in apoptosis. We next tested whether apoptosis or another type of cell death occurred in RTA-induced BCBL-1/off + RTA-2 cells. As shown in Fig. 2B, RTA expression did not induce cell death, and RTA-induced BCBL-1/off + RTA-2 cells grew as well as uninduced ones. The other RTA-inducible BCBL-1 clones also grew as well as uninduced cells (data not shown). Furthermore, the TUNEL assay (Fig. 3B), Annexin V (Fig. 3D), PI staining (data not shown), and Western blotting for PARP cleavage (Fig. 3F) were all negative in the RTA-induced BCBL-1/off + RTA-2 cells. We continued to examine the cells until 13 days postinduction, but no change was observed (data not shown).

These results suggest that RTA has an apoptosis-inducing activity, and that some antiapoptotic mechanism is


BJAB/off+RTA-27
BCBL-1/off+RTA-2
A.
B.
4 days
6 days
8 days
C.
D.
Annexin V
E.
F.
Tc (+)
Tc(-)
(kDa)
(day)
160
105
75
*
**

present in KSHV-infected cells, but not in uninfected BJAB cells, which originate from the B-cell linage as do the PEL-derived BCBL-1 cells.

Discussion

In this article, we showed that KSHV RTA induced apoptosis in a B -cell line, and that the apoptotic pathway was interrupted in KSHV-infected cells. The apoptosis-inducing anti-Fas antibody can induce apoptosis within a few hours (Ashkenazi and Dixit, 1998). KSHV v-cyclin also induces apoptosis when it is transfected into human osteosarcoma cell lines and other cells (Ojala et al., 1999); this apoptosis is detected almost 2 days posttransfection. It took more than 4 days for RTA to induce apoptosis, while RTA expression itself was observed 12 h postinduction, suggesting that the apoptosis induced by RTA expression in uninfected cells and by KSHV v-cyclin takes place by an indirect mechanism, which is stimulated by the stepwise activation of some endogenous cell-suicide genes. RTA is a viral transactivator that triggers the expression of other lytic genes and promotes viral replication ation (Sun et al., 1998; Lukac et al., 1999; Nishimura et al., 2001). RTA is known to upregulate viral gene expression that is both dependent on and independent from specific DNA-binding activity (Lukac et al., 2001; Song et al., 2001; Liang et al., 2002; Ueda et al., 2002). Thus, it is thought that the transcription of several cellular genes is upregulated by RTA expression to produce the equipment needed for viral replication, and it is possible that one of these genes results in the induction of apoptosis, as seen in the BJAB cells, though such genes have not been identified yet. The observation of PARP cleavage in these cells suggested that the apoptosis induced by RTA occurred through a caspase-dependent pathway (Nicholson and Thornberry, 1997; Salvesen and Dixit, 1997).

Gwack et al. reported that RTA represses the transcriptional activity of p53 and p53-induced apoptosis through interaction with CREB-binding protein (CBP) (Gwack et al., 2001). However, the conditional expression of RTA in BJAB/off + RTA-27 and BCBL-1/off + RTA-2 cells did not lead to p53 induction (data not shown), and recent reports show that the KSHV K8 gene product (K-bZip), whose expression is regulated by RTA, induces G_1/S arrest without p53 induction (Wu et al., 2002; Wang et al., 2003). Thus, it is unlikely that a p53-dependent apoptosis pathway was involved in the KSHV-infected cells. Rather, we think it likely that RTA upregulates some other cellular gene expression that is involved in apoptosis and turns on caspase activity in a p53-independent manner.

KSHV has many genes that have an antiapoptotic function, such as KS-bcl-2, v-FLIP, vIRF, and K7 (Cheng et al., 1997; Belanger et al., 2001; Seo et al., 2001; Feng et al., 2002). Such gene products might oppose the cell death induced by RTA during viral replication. Since RTA induces the expression of a number of lytic genes, these proteins are expressed under an RTA-induced condition, and they could coordinately interrupt the apoptotic signal induced by RTA. Recently, we observed that KSHV ORF57 might induce the expression of the antiapoptotic protein XIAP through an IRES-dependent mechanism (K. Nishimura et al., submitted for publication). Thus, XIAP may be an antiapoptotic factor opposing the RTA-induced apoptosis.

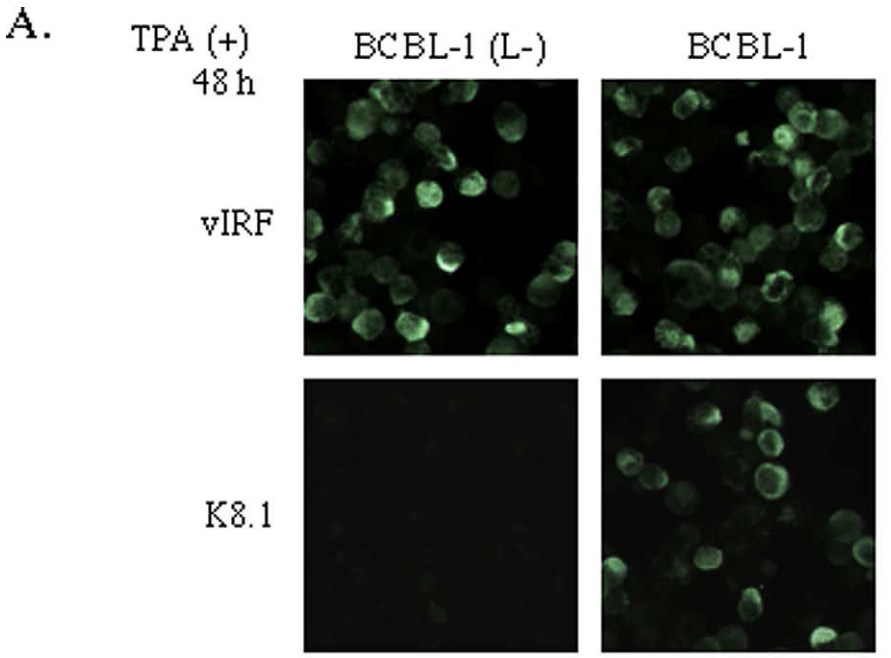
In summary, we showed that RTA was rather a potent apoptosis inducer, apoptosis by which was executed in uninfected cells, but not in replication-incompetent KSHV-infected cells, where the KSHV immediate-early and early genes were expressed normally. These cell lines will be useful for clarifying what gene expressions are controlled by RTA, which will be investigated using DNA chip analysis. We have also tried to isolate RTA-inducible cell lines from replication-competent KSHV-infected cell lines. Such a clone may be used in studies to assess the effect of RTA expression on viral replication and late gene expression and may enable us to obtain cell-free virus particles without using chemical reagents such as TPA.

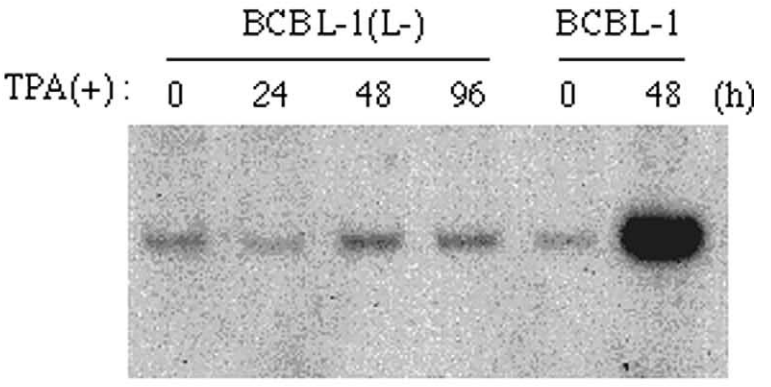
Materials and methods

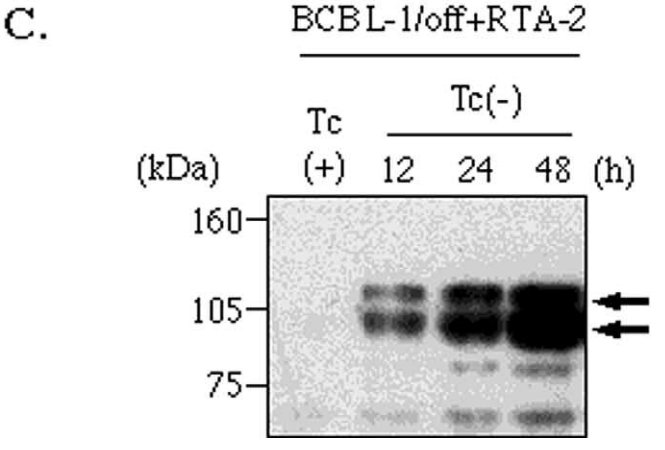
Cell culture

BJAB cells and clones from BJAB cells [BJAB/off and BJAB/off + RTA (see below)] were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and other reagents, G418, hygromycin, and tet, when they were needed, and BCBL-1 cells and clones from BCBL-1 cells [BCBL-1/off and BCBL-1/off + RTA (see below)] were grown with 20% heat-inactivated FBS. RetroPack PT67 packaging cells (Clontech) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FBS.

Fig. 3. RTA-induced apoptosis in BJAB cells, but not BCBL-1 cells. (A, B) Detection of DNA fragmentation in BJAB/off + RTA-27 (A) and BCBL-1/off + RTA-2 (B) cells by theTUNEL assay. RTA-induced cells were collected at the indicated times postinduction and stained as described under Materials and methods. Labeled cells (bold line) were analyzed by flow cytometry using unstained cells (dashed line) as a control. (C, D) Detection of structural changes in the cell membrane of BJAB/off + RTA-27 (C) and BCBL-1/off + RTA-2 (D) by Annexin V staining. Fluorescence from the FITC-Annexin V of induced (bold line) and uninduced (dashed line) cells was detected by flow cytometry. (E, F) Cleavage of poly(ADP-ribose) polymerase (PARP) in BJAB/off + RTA-27 (E) and BCBL-1/off + RTA-2 (F) cells. Cells harvested at different times postinduction were treated and analyzed by SDS–PAGE and Western blotting as indicated under Materials and methods. Full-length PARP (*) and the larger cleavage product (**) were detected by the same monoclonal antibody against PARP. The positions of molecular mass markers are shown on the left.


A.
TPA (+)
48 h
BCBL-1 (L-)
BCBL-1
vIRF
K8.1


B.
BCBL-1(L-)
BCBL-1
TPA(+):
0
24
48
96
0
48
(h)


C.
BCBL-1/off+RTA-2
Tc
(+)
Tc(-)
12
24
48
(h)
(kDa)
160
105
75

To induce the expression of RTA, cells grown in medium containing 0.1 μg/ml tet were collected and washed twice with phosphate-buffered saline (PBS), and then cultured in medium with (uninduced) or without (induced) 0.1 μg/ml tet.

Lytic gene expression was occasionally induced by treatment with TPA at 25 ng/ml in some cases.

To investigate cell viability, the cells were stained with 0.04% trypan blue in PBS, followed by observation under a microscope, and live and dead cells were counted. Viability was expressed as (live cell number)/(live cell number plus dead cell number) × 100 (%).

Plasmids

pcDNA3.1-ORF50-cDNA, described previously (Chen et al., 2000), which encodes the full-length RTA protein with a myc-epitope and a $(His)_6$-tag at the carboxy-terminus, was digested with *Pme*I, and the fragment containing the RTA cDNA sequence was inserted into the *Hpa*I site of the pRev-TRE (Clontech). The resultant plasmid, named pRev-TRE-RTA, was used for the generation of RTA-inducible cells.

Establishment of clones that inducibly produce RTA

For the isolation of stable RTA-inducible cells, we used the RevTet-Off system (Clontech), which combines retrovirus-mediated gene transfer with the tetracycline-regulated control of the Tet-Off system. We first established BJAB cells that stably expressed tTA. RetroPack PT67 packaging cells (Clontech) were transfected with pRevTet-Off (Clontech), and the G418-resistant (0.5 mg/ml) clones were isolated. Titers of the virus produced from the G418-resistant PT67 cell clones were determined by infection of NIH3T3 cells. BJAB cells were infected with tTA-encoding retroviruses collected from PT67 cells producing high titers of virus. BJAB/off cell clones that stably express tTA were selected with medium containing G418 (0.8 mg/ml) and isolated in RPMI 1640 supplemented with 20% FBS and 1.5% methylcellulose no. 4000. To test for inducibility, the BJAB/off cells were transiently infected with virus encoding TRE-Luc, the luciferase gene driven by TRE, and the luciferase activity in the induced and uninduced cells was compared using the luciferase assay system (Promega).

Next, BJAB/off cell clones that showed higher inducibility were infected with the TRE-RTA-encoding retroviruses produced from a PT67 cell clone that was stably transfected with pRev-TRE-RTA. Positive cells were selected with 0.4 mg/ml hygromycin and 1.0 μg/ml tet and cloned. G418- and hygromycin-resistant clones (BJAB/off + RTA) were screened for the inducibility of RTA expression by IFA using specific monoclonal antibodies against RTA. Tet was present at 1.0 μg/ml during clone selection, but its concentration was reduced to 0.1 μg/ml for maintenance. This lower tetracycline concentration allowed a much more rapid induction of the RTA gene, without increasing its basal expression level.

BCBL-1 cell clones in which stable RTA expression was regulated by the Tet-Off system (BCBL-1/off + RTA) were established in the same way as the BJAB/off + RTA cells. Cells that contained only TRE instead of TRE-RTA were similarly established and used as a control.

Immunofluorescence assay (IFA)

At the selected time after induction, the cells were harvested, washed twice with PBS, spotted onto glass slides, air-dried, and fixed with acetone:methanol (50:50) for 10 min at −20°C. The fixed cells were incubated for >12 h at room temperature with a mouse monoclonal antibody (approximately 1.0 μg/ml) against each of the following KSHV-specific antigens: RTA (Nishimura et al., 2001), ORF57 (K. Nishimura et al., submited for publication), K5 (Haque et al., 2000), K8, vIRF, ORF59, and K8.1 (Okuno et al., 2002). After being washed three times with PBS containing 0.05% Tween 20 for 5 min each, the slides were air-dried and incubated with approximately diluted fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibodies (DAKO) for 1 h. After being washed as described above, the slides were mounted with 90% glycerol in PBS and the signals were detected by fluorescence microscopy.

Western blotting

Whole-cell protein extract was prepared from RTA-induced or uninduced cells at 12, 24, and 48 h after induction. The protein concentration of the extracts was determined by the Bradford assay. Samples (20 μg) were subjected to sodium dodecyl sulfate–7.5% polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions and then electrophoretically transferred to polyvinylidene difluoride membranes (Bio-Rad) in 25 mM ethanolamine/NaOH (pH

Fig. 4. Generation of RTA-inducible BCBL-1 cells from replication-deficient BCBL-1 cells. (A) Immunofluorescence assay of KSHV lytic proteins. Replication-deficient BCBL-1 clone (BCBL-1[L−]) and BCBL-1 cells were fixed 48 h after TPA induction and labeled with an anti-vIRF or anti-K8.1 mouse monoclonal antibody, respectively,followed by a fluorescein isothiocyanate-conjugated anti-mouse IgG. Original magnification, ×400. (B) KSHV DNA synthesis in replication-deficient BCBL-1 clone (BCBL-1[L−]) and BCBL-1 cells. Cells were collected at the indicated times after TPA induction, and DNA was extracted from the whole cells. Samples containing the same amount of DNA (5 μg) were analyzed by Southern blotting using a probe specific for the KSHV K8 region as described under Materials and methods. (C) RTA expression in BCBL-1/off + RTA-2 cells. Whole-cell extracts were obtained at different times postinduction (Tc[−]) and analyzed by Western blotting using a specific antibody against RTA. The same amount of total protein (20 μg) was loaded in each lane. The positions of molecular mass markers are shown on the left. The arrow indicates RTA protein.

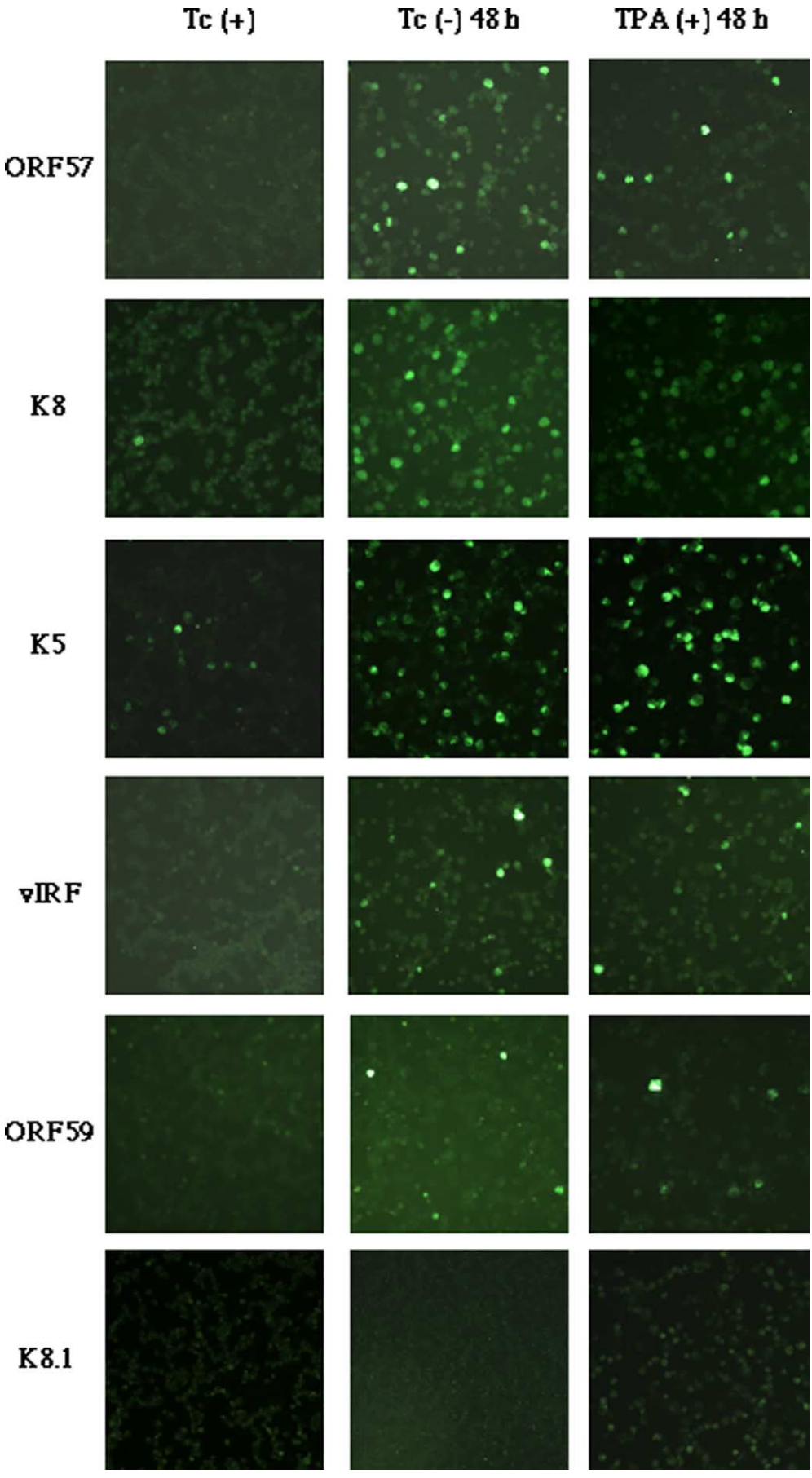


Fig. 5. The expression of KSHV lytic proteins in uninduced, RTA-induced, and TPA-induced BCBL-1/off + RTA-2 cells. Cells were fixed 48 h postinduction and labeled as indicated under Materials and methods with a mouse monoclonal antibody specific for the proteins indicated on the left of the photographs. Original magnification, ×100.

9.5). The membranes were blocked for 1 h while shaking at room temperature in TBS (137 mM NaCl, 2.68 mM KCl, 25 mM Tris) containing 0.05% Tween 20 and 5% dry milk and then incubated with either a mouse anti-RTA or a mouse anti-PARP antibody (Kamiya Biomedical Co.) (the former diluted in TBS–0.05% Tween 20 to 0.1 μg/ml, and the latter diluted to 0.5 μg/ml) for 1 h with shaking at room temperature. The membranes were then washed three times for 5 min each in TBS–0.05% Tween 20 and incubated for 1 h with shaking at room temperature with horseradish peroxidase conjugated donkey anti-mouse IgG antibodies (1:5000 dilution) (Amersham Pharmacia Biotech). After the membranes were washed, the signals were detected using Super Signal West Pico Chemiluminescent substrate (Pierce), according to the manufacturer's instructions.

Southern blotting

Approximately 1×10^7 of BCBL-1 and replication-deficient BCBL-1 cells, untreated or treated with TPA, were lysed in lysis buffer [150 mM NaCl, 10 mM Tris–HCl (pH 8.0), 10 mM EDTA, 0.1% SDS] supplemented with 100 μg/ml proteinase K and incubated at 55°C for 1 h followed by an overnight incubation at 37°C. Cell lysate was extracted twice with phenol/CHCl, and the DNA was precipitated in 70% ethanol. The DNA pellet was dissolved in TE [10 mM Tris–HCl (pH 8.0), 1 mM EDTA (pH 8.0)], and RNase A was added to a final concentration of 100 μg/ml. The samples were incubated at 37°C for 1 h; then the RNase A was removed by phenol/$CHCl_3$ extraction. The DNA was then pelleted, dried, and dissolved in TE. DNA samples (5 μg) were digested with *Bam*HI, fractionated on a 1% agarose gel, and transferred to a Hybond-N^+ membrane under denaturing conditions (0.4 M NaOH). The blots were UV cross-linked using a UV Stratalinker 2400 (Stratagene) with the standard protocol (120 mJ/cm^2) and hybridized with probes specific for the K8 region labeled by the random prime labeling system (Amersham) with [α-^{32}P]dCTP (Amersham), in hybridization buffer (6× SSC, 1% SDS, 100 μg/ml sonicated salmon testis DNA, 1 mg/ml BSA, 1 mg/ml Ficoll 400, 1 mg/ml PVP360).

Detection of apoptosis

For the TUNEL assay, RTA-induced or uninduced cells were washed, fixed, stained, and processed using the In Situ Cell Death Detection Kit (Roche) as described by the manufacturer. Annexin V and PI staining were performed on RTA-induced or uninduced cells using the MEBCYTO-Apoptosis Kit (MBL), according to the manufacturer's instructions. The fluorescence of FITC and PI was detected by FACScan (FACS Caliber, Becton Dickinson).

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